Lipid Supplemented Medium Induces Lamellar Bodies and Precursors of Barrier Lipids in Cultured Analogues of Human Skin

Steven T. Boyce* and Mary L. Williams†

* Shriners Burns Institute and Department of Surgery, University of Cincinnati; and † Department of Dermatology, Veterans Administration Medical Center, and University of California San Francisco, San Francisco, California, U.S.A.

Barrier function of cultured skin substitutes (CSS) is required for their effective use in clinical treatment of skin wounds, and for percutaneous absorption *in vitro*. Arachidonic, palmitic, oleic, and linoleic free fatty acids, in conjunction with the antioxidant alpha-tocopherol acetate (lipid supplements, "LS"), were added to nutrient media of CSS to provide precursors of epidermal barrier lipids. CSS were composed of human keratinocytes (HK), fibroblasts (HF), and collagen-glycosaminoglycan substrates, and were incubated for 14 d submerged or lifted to the air-liquid interface in media based on MCDB 153 ± LS. Duplicate samples (30 cm^2) were harvested and the epidermal analogue was analyzed for total protein, total DNA, total lipid, lipid fractions including acylglucosylceramide (AGC), and presence of lamellar bodies. Significant increases ($p < 0.05$) were detected between CSS incubated in +LS medium for total lipid, total DNA, ceramide, glucosylceramide, triglycerides, and diglycerides. AGC and lamellar bodies were detected only in epithelia of CSS incubated in +LS medium. These data show that free fatty acids, vitamin E, and lifting of CSS promote increased epithelial morphogenesis compared to CSS cultured submerged without lipid supplements. Presence of lamellar bodies and AGC suggests enhanced production *in vitro* of barrier-associated epidermal lipids. *J Invest Dermatol 101:180–184, 1993*

Advances in skin wound treatment [1], alternatives to animals for percutaneous absorption studies [2], and models of human skin biology and pathology [3] have resulted in development of several analogues of human skin prepared *in vitro*. Although most of these analogues demonstrate histologically an epithelial component with cornified strata, barrier function of all of these analogues is significantly deficient compared to native skin [4–6].

The epidermal permeability barrier consists predominantly of intercellular lipids (ceramides, cholesterol, free fatty acids) that behave as liquid crystals according to their physical chemistry [7–9]. Liquid crystal behavior of the lipid fraction of the epidermal barrier is analogous to the fluidity of phospholipid plasma membranes, and therefore is defined in large part by the qualitative and quantitative composition of the intercellular lipids [7] under physiologic conditions of temperature, pressure, and hydration. Because cultured analogues of epidermal skin do not synthesize or release complete profiles of epidermal barrier lipids in the same proportions as native skin [10], it is not surprising that barrier function of these analogues is deficient.

Synthesis and deposition of epidermal barrier lipids is a very complex and highly regulated process that involves metabolic equilibria among carbohydrate metabolism (glycolysis, tri-carboxylic acid cycle); β-oxidation of fatty acids; fatty acid synthesis; uptake and retention of essential fatty acids (e.g., linoleic acid) [11]; and modification of fatty acids to form barrier lipids. In addition, deposition of barrier lipids requires assembly of barrier precursors into lamellar bodies [12] that process enzymatically and transport those lipids to the extracellular space.

Previous studies from this laboratory have described a composite skin substitute consisting of an implantable collagen-glycosaminoglycan (GAG) substrate populated with cultured human epidermal keratinocytes and dermal fibroblasts [13,14]. The present study demonstrates that synthesis of an important precursor of barrier lipids (acylglucosylceramide), and formation of lamellar bodies by cultured epithelium of a skin analogue are induced by addition of free fatty acids (arachidonic, palmitic, oleic, and linoleic acids) and alpha-tocopherol acetate to a serum-free medium for human keratinocytes [15]. Expression of these markers of epidermal barrier by these skin substitutes is independent of lifting to the air-liquid interface, but is enhanced by air exposure. The data suggest that formation *in vitro* of biochemical and ultrastructural markers of epidermal barrier depends on nutritional and environmental regulation of keratinocyte metabolism.

MATERIALS AND METHODS

Media Formulae Basal medium MCDB 153 with 0.5 mM calcium chloride [15,16] and increased concentrations of six amino acids [17] was supplemented as described in Table I. Growth medium (−LS) for keratinocytes contained bovine pituitary extract (0.5% v/v), epidermal growth factor (EGF, 1 ng/ml), insulin (5 μg/ml), and hydrocortisone (0.5 μg/ml). Lipid enriched medium (+LS) contained a) arachidonic, palmitic, oleic, and linoleic free fatty acids (FFA) and alpha-tocopherol acetate; b) fatty-acid free bovine serum albumin (BSA); c) EGF, insulin, hydrocortisone; d) carnitine; and e) increased serine.

Manuscript received September 3, 1992; accepted for publication March 15, 1993.

Presented May 1, 1991 at The 53rd Annual Meeting of the Society for Investigative Dermatology.

Reprint requests to: Dr. Steven Boyce, Research Department, Shriners Burns Institute, 3229 Burnet Avenue, Cincinnati, OH 45229-3095.

Abbreviations: AGC, acylglucosylceramide; CSS, cultured skin substitute; FFA, free fatty acids; LS, lipid supplement; L, lifted; S, submerged.

0022-202X/93/$06.00

Table I. Formulae of Media Supplements

Basal Medium	Supplement	−LS	+LS
MCDB 153[a]	Bovine pituitary extract	0.5% (v/v)	0
	Epidermal growth factor	1 ng/ml	10 ng/ml
	Palmitic acid	0	2.5×10^{-5} M
	Oleic acid	0	2.5×10^{-5} M
	Linoleic acid	0	1.5×10^{-5} M
	Arachidonic acid	0	0.7×10^{-5} M
	dl-α-Tocopherol-Ac	0	2.2×10^{-5} M
	BSA (FFA free, as carrier)	0	2.4×10^{-5} M
	L-carnitine	0	1.0×10^{-5} M
	L-serine	0	1.0×10^{-2} M

[a] Basal medium contained elevated concentrations of six amino acids [17], and 0.5 mM calcium chloride.

Composite Skin Substitutes (CSS), Preparation, and Incubation CSS were prepared in duplicate experiments as previously described [13,18]. Collagen-GAG substrates were inoculated with human dermal fibroblasts (5×10^5/cm²) on the porous surface, followed the next day by human epidermal keratinocytes (1×10^6/cm²) on the non-porous surface [13]. With inoculation of keratinocytes as day 0, skin substitutes were incubated 2 d submerged in keratinocyte growth medium (−LS, Table I), and then two skin substitutes (72 cm² each) were incubated submerged in −LS and two in +LS medium from days 2–4. All media were changed daily. On day 5, one skin substitute in each medium remained submerged (S), one in each medium was lifted (L) to the air-liquid interface, and skin substitutes in all four conditions (S ± LS, L ± LS) were incubated until day 14.

Data Collection and Analysis CSS samples (30 cm² each) were collected from duplicate experiments at day 14 after inoculation of keratinocytes, and processed for determinations of total contents of DNA, lipid, and protein [19], and lipid fractions (neutral lipids, sphingolipids) by thin-layer chromatography. Epithelia were released from CSS by treatment with Dispase, centrifuged at 220 × *g* and resuspended in 1.2 ml Hoescht buffer. Samples were homogenized by brief sonication, and divided into duplicate aliquots for determination of DNA (2 × 100 μl), protein (2 × 100 μl), and lipid (2 × 400 μl). DNA content was determined using fluorescence enhancement by staining with bisbenzimidazole [20]. Protein was measured by direct binding of Coomassie Brilliant Blue G-250 [21]. Lipids were extracted according to the method of Bligh and Dyer [22]. Lipids were fractionated by high-performance thin layer chromography (TLC) as previously described [23]. Briefly, lipid extracts were solubilized in chloroform, and applied in 5-mm lanes (30–60 μg/lane) on 10 × 20 cm high-performance TLC plates using a CAMAG Linomat IV autospotter. Authentic standards in 1.0 μg quantities flanked both ends of the plate to establish standard for densitometry. Sequential development systems for separation of neutral lipids and sphingolipids have been described in detail elsewhere [23]. To summarize, neutral lipids were developed in petroleum ether : diethylether : acetic acid (80 : 20 : 1 vols). Sphingolipids were subfractionated by sequential development in three solvent systems: System 1, chloroform : methanol : acetone (76 : 20 : 4 vols), developed first to 10 mm, then to 35 mm; system 2, chloroform : acetone : methanol (80 : 10 : 10 vols) to 75 mm; and system 3, chloroform : ethylacetate : diethylether : methanol (76 : 20 : 6 : 2 vols). After development, plates were dipped in charring solution and heated to 180°C for 15 min in a mechanical convection oven. Lipid bands were quantitated with a recording photo-densitometer equipped with automatic peak integration in reference to the closest migrating ceramide standard [23].

Cell-cycle analysis was performed by flow cytometry on keratinocyte nuclei (2×10^4/sample) labeled with propidium iodide [24] from lifted cultures only. Cell population subsets were calculated directly by a computer program based on the Dean and Jett model of analysis [25]. Histology and transmission electron microscopy were performed by routine techniques [26].

Quantitative data were evaluated by two-way analysis of variance for significant differences ($p < 0.05$) among groups. χ^2 analysis was used to test whether the lipid supplement was related to significant differences among experimental conditions.

Table II. Biochemical Fractions[a]

Fraction	S −LS	S +LS	L −LS	L +LS
Total lipid (mg)	1142 ± 447	1789 ± 12.0	1414 ± 166	2677 ± 228
Total protein (mg)	0.95 ± 0.01	0.74 ± 0.05	0.93 ± 0.07	1.13 ± 0.24
Total DNA (mg)	134 ± 8.41	235 ± 19.2	135 ± 9.01	223 ± 6.60
Lipid : DNA	8.32 ± 2.80	7.65 ± 0.57	10.6 ± 1.94	12.0 ± 1.37
Sphingolipids (μg)				
Ceramide	46.5 ± 7.50	84.2 ± 16.7	63.9 ± 8.55	106 ± 16.2
Glucosyl-ceramide	7.10 ± 0.80	14.3 ± 1.70	8.15 ± 1.85	23.1 ± 6.00
AGC	0 ± 0	2.50 ± 1.10	0 ± 0	3.90 ± 1.90
Neutral lipids (μg)				
Triglycerides	354 ± 116	704 ± 27.1	357 ± 64.9	1012 ± 33.2
Free sterols	163 ± 43.4	186 ± 16.6	154 ± 65.6	370 ± 71.4
Free fatty acids	39.6 ± 9.70	36.8 ± 19.0	47.6 ± 4.85	38.6 ± 15.4
Diglycerides	8.70 ± 2.40	15.3 ± 4.55	9.90 ± 1.20	26.3 ± 3.10
Sterol esters	8.00 ± 3.10	12.5 ± 0.10	9.55 ± 1.65	17.5 ± 2.80

[a] Values expressed as mean ± SEM wt/30 cm² duplicate samples.

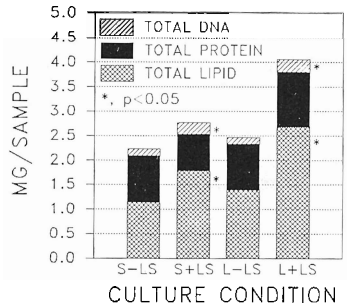

Figure 1. Plot of keratinocyte total lipid, DNA, and protein versus culture condition. Conditions consisted of skin substitutes incubated submerged (S) in medium, or lifted (L) to the air-liquid interface, in medium with lipid supplement (+LS) or without (−LS). Skin substitutes incubated in medium +LS show increased total lipid and DNA contents compared to samples without lipid supplement.

RESULTS

Biochemical Fractions Table II summarizes quantitative data of biochemical fractionation. Figure 1 shows differences in total sample contents of DNA, lipid, and protein among the four culture conditions. Total DNA and lipid are significantly greater ($p < 0.05$) in +LS medium whether submerged or lifted, and are highest in lifted in +LS medium. Figure 2 shows that ratios of μg lipid/μg DNA are increased in lifted cultures compared to submerged, and

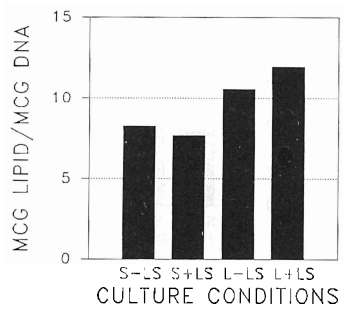

Figure 2. Plot of ratio of lipid : DNA versus culture condition. Conditions as described in Fig 1. Increased proportion of lipid : DNA is suggested to result from lifting of CSS to the air-liquid interface, and not only from addition of lipid supplement to the culture medium. CSS that were lifted +LS exhibited the greatest lipid : DNA ratio of the conditions tested.

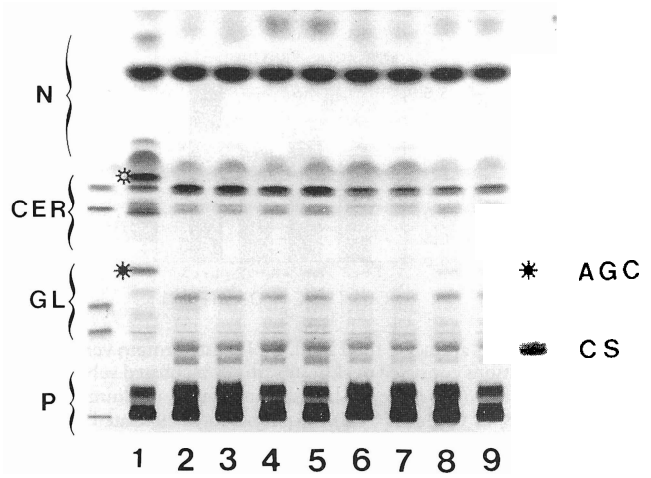

Figure 3. High-performance TLC of sphingolipid fractions from keratinocytes in ±LS medium and with or without lifting. Neutral lipids (N), ceramides (CER), glycolipids (GL), and phospholipids (P) are indicated. AGC are identified with *closed asterisks;* acylceramides are identified with an *open asterisk. Leftmost lane,* sphingolipid standards. *Lane 1,* stratum corneum lipids isolated from murine epidermis. *Lanes 2* and *3,* submerged culture −LS. *Lanes 4* and *5,* submerged culture +LS. *Lanes 6* and 7, lifted culture −LS. *Lanes 8* and *9,* lifted culture +LS. *Rightmost lane,* cholesterol sulfate standard.

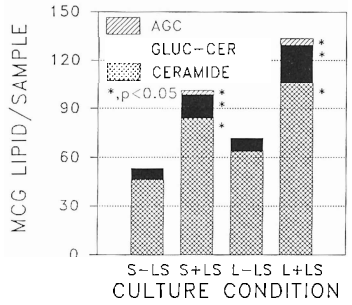

Figure 4. Plot of subfractionation of sphingolipids versus culture condition. Conditions as described in Fig 1. Addition of lipid supplement to the culture medium stimulates increased appearance of ceramide and glucosylceramide. Acylglucosylceramide (AGC) is detected only in samples cultured with lipid supplement.

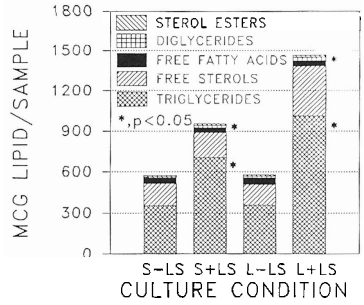

Figure 5. Plot of subfractionation of neutral lipid classes versus culture condition. Conditions as described in Fig 1. Triglycerides, free sterols, free fatty acids, diglycerides, and sterol esters are detected in each of the four conditions tested. Samples +LS contained increased amounts of triglycerides, and decreased free fatty acids compared to identical samples −LS. Presence of the albumin in medium +LS may account, in part, for reduced detection of fatty acids.

Table III. Keratinocyte Cell Cycle Subsets[a]

Medium	G_1	S	$G_2 + M$
−LS	56.0	30.5	13.5
+LS	64.9	22.1	13.1

[a] Percent total nuclei, 2×10^4 nuclei/sample.

also are highest in samples that are lifted in +LS medium. However, no statistically significant differences are detected.

Lipid Fractions Figure 3 shows a thin-layer chromatogram developed for resolution of sphingolipids. Lane 1 contains murine epidermal lipids for comparison; the acylceramide and acylglucosylceramide (AGC) fractions are indicated by open and closed asterisks, respectively. Bands in lanes 4, 5, 8, and 9 are identified as AGC by co-migration with authentic AGC of extracted stratum corneum lipids (lane 1). No other lipids migrate to this relative position in the solvent separation systems used [23]. Figure 4 illustrates the amounts of ceramide, glucosylceramide, and AGC recovered from the culture systems. Significantly increased ($p < 0.05$) quantities of the ceramide and glucosylceramide fractions with clear detection of AGC were present after culture in +LS medium. Detection of AGC is independent of submerged or lifted culture condition, and related significantly ($p < 0.05$) to +LS according to χ^2 analysis. Figure 5 compares neutral lipid subfractions. Free fatty acid levels are higher in −LS medium, but not significantly different. Triglycerides are significantly higher in +LS medium, independent of air exposure. However, triglycerides are elevated by 40% or more in lifted cultures in +LS medium compared to all other groups. Diglycerides were also significantly elevated in +LS medium, but no differences were seen in free fatty acids, free sterols, or sterol esters.

Cell-Cycle Analysis Table III shows findings of cell-cycle analysis performed on keratinocyte nuclei isolated from lifted CSS incubated in medium ±LS. No significant differences are detected. Keratinocytes from CSS +LS medium have subsets of 64.9% G_1, 22.1% S, and 13.1% $G_2 + M$. Nuclei from −LS medium have subsets of 56.0% G_1, 30.5% S, and 13.5% $G_2 + M$. Both conditions demonstrate >20% of nucleated keratinocytes in S-phase after 4 d submerged and 10 d lifted culture.

Histology and Transmission Electron Microscopy (TEM) Figure 6 compares light and transmission electron micrographs of samples incubated in these four test conditions. Medium +LS results in improved epithelial organization in lifted (Fig 6*A*) or submerged (Fig 6*E*) culture compared to corresponding samples in −LS medium (Fig 6*C,D*). Samples lifted in +LS medium (Fig 6*A*) have cuboidal keratinocytes attached to the collagen-GAG substrate with flattened cellular strata distal to the attachment surface. Lamellar bodies are found easily after lifted culture +LS (Fig 6*B*), or with less frequency after submerged culture in +LS medium (Fig 6*F*). No lamellar bodies are found in lifted or submerged CSS from −LS medium (not shown).

DISCUSSION

Data from this study demonstrate the well established principles of nutritional and physico-chemical regulation of proliferation and differentiated phenotype of human cells in culture [27]. Metabolic responses including total contents of lipid, DNA, and protein are dependent both on nutritional factors and lifting, and are additive. Synthesis of barrier-specific markers, lamellar bodies (LB), and AGC are also expressed in response to this lipid supplemented medium with increased response in lifted versus submerged cultures. Because the supplement contains linoleic acid, an essential fatty acid and constituent of AGC, it is possible that linoleic acid is a dependent factor in formation of native epidermal barrier. In essential fatty acid deficiency (EFAD), oleic acid substitutes for linoleic acid in epidermal AGC [28]. This may account for the membrane structure abnormalities and barrier defect in EFAD [29,30]. Further-

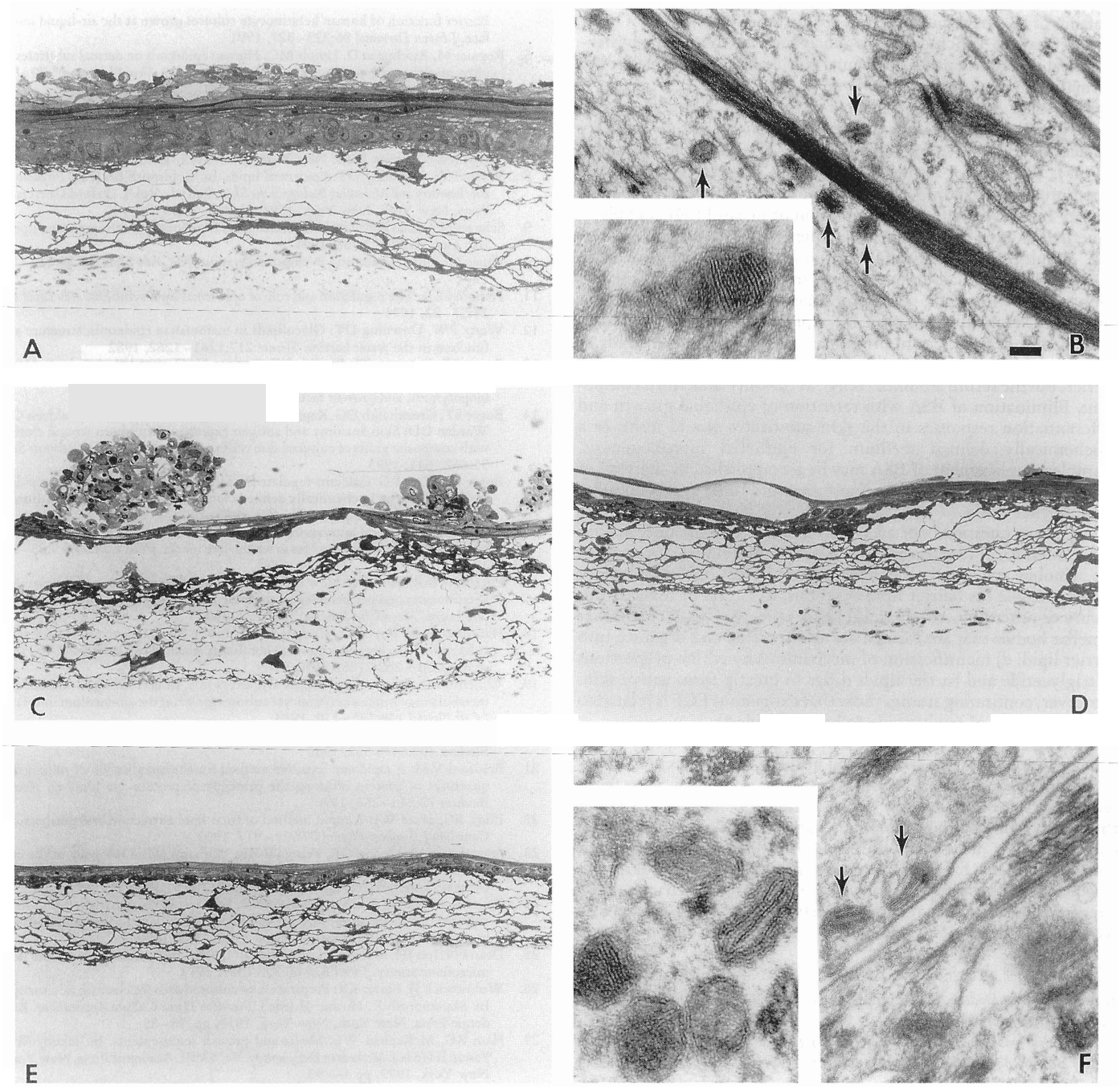

Figure 6. Light and electron micrographs of representative samples. *A, C, D, E;* histologies, *scale bar,* 0.1 mm. *B, F;* TEM, *scale bar,* 0.1 μm. *A*; lifted CSS + LS medium with cuboidal keratinocytes attached to the collagen-GAG substrate and well stratified suprabasal layers. *B*, lamellar bodies occur with high frequency subjacent to cornified cells. *C*, lifted CSS − LS medium shows flattened epithelial morphology, detachment of keratinocytes, and poorly organized epidermal strata. Lamellar bodies are not detected. *D*, submerged − LS culture shows nucleated cells attached to substrate with flattened cells above. *E*, submerged + LS culture shows cuboidal cells attached to substrate with flattened, nucleated cells above. *F*, spherical organelles in the size range of lamellar bodies are seen, but they have poor morphology compared to CSS lifted + LS.

more, pulse-chase experiments have shown transfer of linoleic acid predominantly into triglyceride, but not sphingolipids, of air-exposed epithelial cultures [31,32]. Those findings agree with this study in which the quantity of AGC is much lower than expected (see Fig 3, *lane 1*), and in which linoleic acid is not limiting to AGC synthesis. Rather, it is probable that some or most of the added linoleic acid was incorporated into triglyceride, although linoleic acid content in triglyceride was not determined.

Major aberrations of carbohydrate-lipid metabolism are reflected by excessive accumulations of tri-glyceride and lipid vacuoles within cultured keratinocytes, and by incomplete complement of barrier lipids [33]. Ponec and co-workers have demonstrated these aberrations in native skin after incubation *in vitro,* and conclusively attributed these metabolic errors to the culture environment [10]. Those findings, and results of this study, identify major shifts in metabolic equilibria among glycolysis and tri-carboxylic acid cycle, fatty acid synthesis, β-oxidation of fatty acids, and specific synthesis of complex glyco-lipids and proteo-lipids of the epidermal barrier. Furthermore, it is also known that these metabolic shifts are reversible, because complete epidermal barrier function is restored after

grafting of cultured epithelium to open wounds [13,14,18]. Therefore, it may be concluded that deficiencies in the total physicochemical environment for epidermal culture are responsible for abnormal epidermal physiology *in vitro*.

Growth of human keratinocytes in biochemically defined medium is extended and enhanced by addition of bovine pituitary extract (BPE). This extract contains basic fibroblast growth factor (bFGF), many other growth factors, and hormones, lipids, and blood proteins including albumin. Efforts to purify the mitogenic activity in BPE have led to the isolation of inositol [34], and phosphoethanolamine [35] that are contained in basal medium MCDB 153. Results of this study suggest that other components of BPE, namely, lipids and albumin, may be replaced with their purified counterparts to provide completely the mitogenic activity of the crude tissue extract. In addition, any lipids and albumin supplied by in BPE are not sufficient to promote formation of barrier lipids or lamellar bodies. The formulation described here contains a lipid carrier, bovine serum albumin (BSA), as the only undefined component. Elimination of BSA with retention of epithelial growth and differentiation responses in the skin substitutes would result in a biochemically defined medium for epithelial morphogenesis. Complete replacement of BSA may be accomplished by delivery of lipids as vesicular micelles, or liposomes, and these approaches deserve further study.

Important questions not addressed in this study include, but are not limited to, a) identification of whether individual components (i.e., linoleic acid) or their concentrations in this lipid supplement are responsible for barrier lipid synthesis; b) effects of reduced humidity or other physico-chemical factors (i.e., % CO_2); c) whether lamellar bodies that are formed are also secreted and organize into barrier lipid; d) identification of mechanisms by which proportions of triglyceride and barrier lipids differ so greatly from native skin. However, continuing studies show that exogenous EGF is related to abnormal ratios of triglyceride to barrier lipids.‡

Cultured analogues of human skin have advanced rapidly in design and applications to skin wound healing, study of skin biology, and alternatives to animal testing. Continued progress in these fields will be served by availability of a material of defined composition that is physiologically homologous with native skin. Although no current models of cultured skin§ [4–6,13,36] have demonstrated complete validity for direct comparison to native human skin, the cultured skin substitute described here offers a well-characterized alternative model for research, safety testing, and therapeutic wound treatment.

We thank JoAnn Dodick, Chip Childress, and Andy Supp for technical assistance with the performance of this study.

This work was supported by Shriners Hospitals for Crippled Children, grants 15893 and 15837 (STB); and National Institutes of Health grants AR 29908 and AR 39448 (MLW).

REFERENCES

1. Carver N, Leigh IM: Keratinocyte grafts and skin equivalents. *Int J Dermatol* 30:540–551, 1991
2. Bronaugh RL, Maibach HI: *In vitro* percutaneous absorption. In: Marzulli FN, Maibach HI (eds.). *Dermatotoxicology*, 3rd ed. Hemisphere Publishing Corporation, New York, New York, 1987, pp 121–132
3. Boyce ST, Hansbrough JF, Norris DA: Cellular responses of cultured human epidermal keratinocytes as models of toxicity to human skin. In: Vol 6. Goldberg AM, Principe ML (eds.). *Progress in* In Vitro *Toxicology*, Mary Ann Liebert, Inc., New York, New York, 1988, pp 27–37
4. Mak VH, Cumpstone MB, Kennedy AH, Harmon CS, Guy RH, Potts RO: Barrier function of human keratinocyte cultures grown at the air-liquid interface. *J Invest Dermatol* 96:323–327, 1991
5. Regnier M, Asselineau D, Lenoir MC: Human epidermis on dermal substrates in vitro: an alternative to animals in skin pharmacology. *Skin Pharmacol* 3:70–85, 1990
6. Parenteau NL: Epidermis generated *in vitro:* practical considerations and applications. *J Cell Biochem* 45:245–251, 1991
7. Small DM: *The Physical Chemistry of Lipids; From Alkanes to Phospholipids.* Small DM (ed.). Plenum Press, New York, New York, 1986
8. Wertz PW, Downing DT: Epidermal lipids. In: Goldsmith LA (ed.). *Physiology, Biochemistry and Molecular Biology of the Skin*, 2nd ed., Vol. I. Oxford University Press, New York, New York, 1991, pp 205–236
9. Schurer NY, Elias PM: The biochemistry and function of stratum corneum lipids. *Adv Lipid Res* 24:27–56, 1991
10. Ponec M: Lipid metabolism in cultured keratinocytes. *Adv Lipid Res* 24:83–118, 1991
11. Feingold KR: The regulation and role of epidermal lipid synthesis. *Adv Lipid Res* 24:57–82, 1991
12. Wertz PW, Downing DT: Glycolipids in mammalian epidermis: structure and function in the water barrier. *Science* 217;1261–1262, 1982
13. Boyce ST, Foreman TJ, English KB, Stayner N, Cooper ML, Sakabu S, Hansbrough JF: Skin wound closure in athymic mice with cultured human cells, biopolymers, and growth factors. *Surgery* 110:866–876, 1991
14. Boyce ST, Greenhalgh DG, Kagan RG, Housinger TA, Rieman M, Childress CP, Warden GD: Skin anatomy and antigen expression after burn wound closure with composite grafts of cultured skin cells and biopolymers. *Plast Reconstr Surg* 91:632–641, 1993
15. Boyce ST, Ham RG: Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and free serial culture. *J Invest Dermatol* 81(suppl 1):33S–40S, 1983
16. Boyce ST, Ham RG: Cultivation, frozen storage, and clonal growth of normal human epidermal keratinocytes in serum-free media. *J Tiss Cult Meth* 9:83–93, 1985
17. Pittelkow MR, Scott RE: New techniques for the *in vitro* culture of human skin keratinocytes and perspectives on their use for grafting of patients with extensive burns. *Mayo Clin Proc* 61:771–777, 1986
18. Hansbrough JF, Boyce ST, Cooper ML, Foreman TJ: Burn wound closure with cultured human keratinocytes and fibroblasts attached to a collagen-GAG substrate. *JAMA* 262:2125–2130, 1989
19. Williams ML, Brown BE, Monger DJ, Grayson S, ELias PM: Lipid content and metabolism of human keratinocyte cultures grown at the air-medium interface. *J Cell Physiol* 136:103–110, 1988
20. Labarca C, Paigen K: A simple, rapid and sensitive DNA assay procedure. *Anal Biochem* 102:344–352, 1980
21. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt Biochem* 72:248–254, 1976
22. Bligh EG, Dyer WJ: A rapid method of total lipid extraction and purification. *Canadian J Biochem Physiol* 37:911–917, 1959
23. Aszterbaum M, Menon GK, Feingold KR, Williams ML: Ontogeny of the epidermal barrier to water loss in the rat: correlation of function with stratum corneum structure and lipid content. *Pediatr Res* 31:308–317, 1992
24. Boyce S, Michel S, Reichert U, Shroot B, Schmidt R: Reconstructed skin from cultured human keratinocytes and fibroblasts on a collagen-glycosaminoglycan biopolymer substrate. *Skin Pharmacol* 3:136–143, 1990
25. Dean PN, Jett JH: Mathematical analysis of DNA distributions derived from flow microfluorometry. *J Cell Biol* 60:523–527, 1974
26. Wolosewick JJ, Porter KR: Preparation of cultured cells for electron microscopy. In: Maramorosh K, Hirumi H (eds.). *Practical Tissue Culture Applications.* Academic Press, New York, New York, 1979, pp. 58–85
27. Ham RG, McKeehan WL: Media and growth requirements. In: Jakoby WB, Pastan IH (eds.). *Methods in Enzymology.* Vol LVIII. Academic Press, New York, New York, 1979, pp 44–93
28. Wertz PW, Cho ES, Downing DT: Effect of essential fatty acid deficiency on the epidermal sphingolipids of the rat. *Biochim Biophys Acta* 753:350–355, 1983
29. Hartop PJ, Protty C: Changes in the transepidermal water loss and the composition of epidermal lecithin after applications of pure fatty acid triglycerides to the skin of essential fatty acid-deficient rats. *Br J Dermatol* 95:255–264, 1976
30. Elias PM, Brown B, Ziboh VA: The permeability barrier in essential fatty acid deficiency: evidence for a direct role for linoleic acid in the epidermal barrier function. *J Invest Dermatol* 74:230–233, 1980
31. Shurer NY, Monger DJ, Hincebergs M, Williams ML: Fatty acid metabolism in human keratinocytes cultivated at the air-medium interface. *J Invest Dermatol* 92:196–202, 1989
32. Bodde HE, Holman B, Spies F, Weerheim A, Kempenaar J, Mommaas M, Ponec M: Freeze-fracture electron microscopy of *in vitro* reconstructed human epidermis. *J Invest Dermatol* 95:108–116, 1990
33. Ponec M, Weerheim A, Kempenaar J, Mommaas A-M, Nugteren DH: Lipid composition of cultured human keratinocytes in relation to their differentiation. *J Lipid Res* 29:949–961, 1988
34. Gordon PR, Gilchrest BA: Inositol is a required nutrient for keratinocyte growth. *J Cell Physiol* 135:416–424, 1988
35. Kano-Sueoka T, Cohen DM, Yamaizumi Z, Nishimura S, Mori M, Fujiki H: Phosphoethanolamine as a growth factor of mammary carcinoma cell line of rat. *Proc Natl Acad Sci USA* 76:5741–5744, 1979
36. Bell E, Ehrlich HP, Buttle DJ, Nakasuji T: A living tissue formed *in vitro* and accepted as a full thickness skin equivalent. *Science* 211:1042–1054, 1981

‡ Boyce ST, Hoath SB, Wickett RR, Harriger MD, Williams ML: Loss of EGF requirement by cultured analogues of normal human skin: biochemical and physiologic analyses (abstr). *J Invest Dermatol* 100:579A, 1993.

§ Slivka SR, Landen L, Zimber MP, Bartel RL: Biochemical characterization of barrier function and drug metabolism in an *in vitro* skin model (abstr). *J Cell Biol* 115(3):236a, 1991.